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Mdr1b facilitates p53-mediated cell death and p53 is required for *Mdr1b* upregulation *in vivo*

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The mdr1b gene is thought to be a "stress-responsive" gene, however it is unknown if this gene is regulated by p53 in the whole animal. Moreover, it is unknown if overexpression of mdr1b affects cell survival. The dependence of mdr1b upon p53 for upregulation was evaluated in p53 knockout mice. Wild-type (wt) or p53−/− mice were treated singly or in combination with gamma irradiation (IR) and/or the potent DNA damaging agent, diethylnitrosoamine (DEN). Both IR and DEN induced *mdr1b* in wild-type animals, but not in the p53−/− mice. IR also upregulated endogenous *mdr1b* in the H35 liver cell line, and the *mdr1b* promoter was activated by IR and activation correlated with p53 levels; moreover activation required an intact p53 binding site. Colony survival studies revealed that co-transfection of both mdr1b and p53 dramatically reduced colony numbers compared to cells transfected with either p53 or mdr1b alone and cells microinjected with both mdr1b and p53 had a more dramatic loss in viability compared to cells injected with either expression vector alone. Further studies using acridine orange and ethidium bromide to measure apoptosis revealed that mdr1b caused apoptosis and this was enhanced by p53, however the increased apoptosis required a functional p53 transactivation domain. These studies indicate that mdr1b is a downstream target of p53 in the whole animal and expression of mdr1b facilitates p53-mediated cell death. *Oncogene* (2001) **20**, 303–313.

Keywords: Apoptosis; MDR; diethylnitrosamine

Introduction

Mammals possess plasma membrane ATP dependent transporters that can confer resistance to structurally diverse amphipathic cytotoxic agents (Gottesman and Pastan, 1993). While the human *MDR1* gene represents the prototype member, genes that are highly related have been cloned from rodents (Gottesman and Pastan, 1993). The rodent *mdr1a* and *mdr1b* genes share greater than 88% identity, are often expressed in the same tissues and can be selectively overproduced during selection for resistance to cytotoxic agents (e.g., taxol, vinblastine, adriamycin (Lothstein *et al.*, 1989)). Further, selection for drug resistance with ectopically introduced *mdr1a* or *mdr1b* expression vectors yields similar drug resistance profiles for many drugs (Devault and Gros, 1990). In contrast, the application of cytotoxic drug selective pressure to murine macrophage cells caused a switch in endogenous mdr expression from predominantly *mdr1b* to *mdr1a* (Lothstein *et al.*, 1989; Lin *et al.*, 1995). The basis for the switch is a presumed transcriptional upregulation of *mdr1a*. Although, the mechanism accounting for this conversion is unknown, it may be hypothesized that high levels of mdr1b are deleterious to cell survival, a concept that is consistent with studies showing that cytotoxic drugs that are not mdr1 substrates as well as oxidative stress induce *mdr1b* (Ziemann *et al.*, 1999; Thevenod *et al.*, 2000) and that transcriptional upregulation of mdr1b has been correlated with decreased viability (Schrenk *et al.*, 1996).

Drug sensitivity studies reveal, with respect to drug substrates, almost identical functions between mdr1a and mdr1b (Lothstein *et al.*, 1989). This redundancy in drug transport seems unnecessary, especially considering that both genes are co-expressed in many tissues, and in some cases at similar levels (e.g., liver, adrenal, colon and lung) (Schinkel *et al.*, 1995). These findings suggest that mdr1a and mdr1b have additional biological functions independent of drug transport. Indeed, recent studies revealed functional differences between mdr1a and mdr1b (Valverde *et al.*, 1996). In particular, mdr1a increases the rate of activation of swelling-activated chloride channels in response to hypo-osmotic conditions, thereby facilitating restoration of cell volume after exposure to hypo-osmotic stress (Valverde *et al.*, 1996) (human MDR1 is mdr1a's functional orthologue in this respect). In contrast, it has been reported that cells overexpressing mdr1b have an impaired ability to activate chloride channels in response to hypo-osmotic conditions (Bond *et al.*, 1998). Further studies by several groups have suggested that MDR1 plays a role in apoptosis. For example, ectopic overexpression of MDR1 diminishes the

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apoptotic response induced by growth factor withdrawal (Robinson *et al*., 1997) decreases complement mediated cytotoxicity (Weisburg *et al*., 1999) and impairs the activation of caspase-dependent cell death pathways (Kojima *et al*., 1998; Smyth *et al*., 1998). In contrast, it is unknown if mdr1b overexpression affects cell viability.

Wildtype p53 is a transcription factor that plays a critical role as a transducer of cellular stresses in response to environmental cues (Levine, 1997). Many transcriptional targets of p53 have biological roles in either cell cycle arrest (e.g., p21 or gadd45 (Levine, 1997)), regulating p53 (e.g., mdm2) (Momand *et al*., 1992) or modulating apoptosis (e.g., BAX, PIG3). Transcriptional activation by p53 requires at least two copies of the specific DNA binding consensus site (rrrCwwGyyy (Zambetti and Levine, 1993)) located either upstream or downstream of transcriptional initiation (e.g., p21, BAX, mdm2 (Zambetti and Levine, 1993)). The cellular response to elevated p53 levels as either G1 arrest or apoptosis is cell-type dependent (Levine, 1997). P53 also suppresses gene transcription (Zambetti and Levine, 1993) and this requires interactions with TAFs and co-repressors (Murphy *et al*., 1999). One endogenous target of p53 mediated repression, confirmed by both transient transfection assays and inactivation of endogenous p53 is the human *MDR1* and rat *mdr1a* (Thottassery *et al*., 1997).

Although a previous study suggested in cell culture that the rat *mdr1b* gene contained a p53 site it was unclear if this p53 site was relevant to the whole animal from these cell culture studies because: (i) the apparent p53 effects were modest (Zhou and Kuo, 1998), (ii)

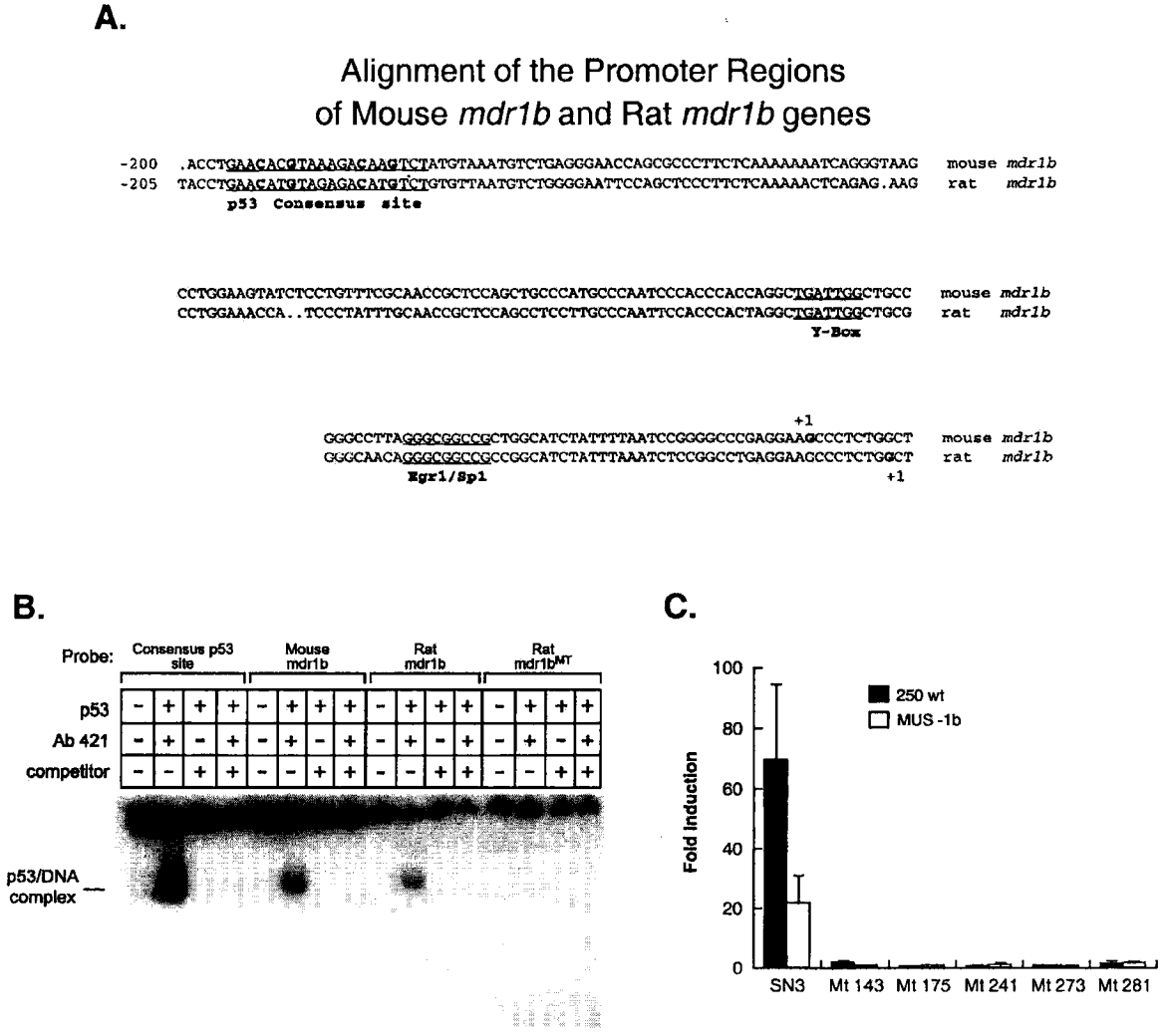

Figure 1 The rodent *mdr1b* genes contain a functional conserved p53 DNA binding site. (**a**) Sequence alignment between mouse and rat *mdr1b*; (**b**) Rat and mouse mdr1b bind recombinant p53 in the presence of Ab421 with binding abrogated by specific mutations and competitors. (**c**) Activation of the rat and mouse *mdr1b* promoters requires wildtype p53 s. The bars represent the average value plus one standard deviation ($n=3-4$ separate experiments with duplicate determinations)

authentic p53 had not been demonstrated to bind the p53 site and (iii) the p53 site functionally overlapped with a NF-κb site that also positively regulated *mdr1b* (Zhou and Kuo, 1997). To approach these issues we used a mouse model to determine if the *mdr1b* gene was regulated by p53 in the whole animal. The p53 nullizygous animals provided an ideal opportunity to determine if mdr1b was regulated by DNA damage. We found that p53 is required for upregulation of mdr1b in the whole animal because the potent DNA damaging agent, diethylnitrosamine (DEN) only upregulated mdr1b in p53+/+ mice and not in p53−/− mice. Furthermore, because mdr1b is a downstream target of p53 we further determined that mdr1b expression in p53-null cells caused decreased cell survival and co-expression of mdr1b and wt p53 indicated p53 cooperated with mdr1b in decreasing cell survival. These studies are the first to demonstrate that p53 plays an essential role in regulating mdr1b in the whole animal and that mdr1b enhances p53's ability to decrease cell survival.

Results

Rodent mdr1b genes contains a conserved, functional p53 site required for optimal basal activity

DNA sequence analysis of the mouse and rat *mdr1b* genes revealed remarkable similarity between the two genes (92%) and their regulatory sequences (e.g., Y-box, Sp1/Egr-1 (Thottassery *et al.*, 1999)). Further analysis revealed both genes contained, in almost identical position, two adjacent p53 sites (rrrCwwGyyy) (Figure 1a). In contrast, the mouse *mdr1a*, rat *mdr1a* genes and human MDR1 do not have p53 binding sites (unpublished). To determine if the positionally conserved p53 site in the *mdr1b* promoters bound authentic p53, gel shift analysis was performed using oligonucleotides to either a p53 consensus site (Nagaich *et al.*, 1997a), the mouse *mdr1b* p53 site, rat *mdr1b* p53 site, or a mutant rat *mdr1b* with nucleotide substitutions at positions 4 and 7 that eliminate p53 DNA binding (the 'C' and 'G' at

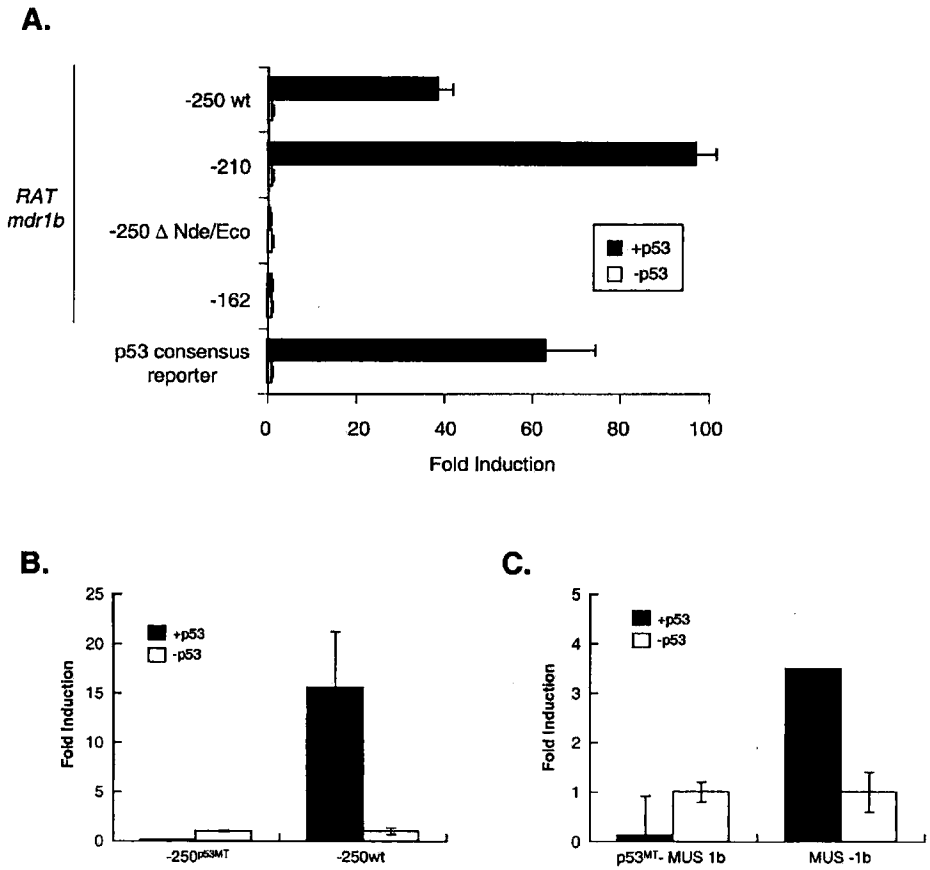

Figure 2 Activation of the rodent *mdr1b* promoters by p53 requires a p53 binding site. (**a**) The mdr1b promoter (−250 to +150) was systematically deleted by either PCR or convenient restriction sites. The reporter, p50-2, that contains two consensus p53 binding sites fused to a TATA-inr was used as a positive control for p53 activation. (**b**) The p53 site in the rat *mdr1b* promoter was mutated (see Materials and methods) and the reporters containing either the wild-type *mdr1b* promoter or the promoter with the mutated mdr1b p53 site were transfected into Saos-2 cells in either the presence or absence of p53. (**c**) The murine *mdr1b* promoter was either intact or the p53 site deleted followed by transfection as described above

positions 4 and 7) (Nagaich *et al.*, 1997b). Because recombinant p53 proteins do not bind p53 sites, due to conformational restraints (Levine, 1997), we used an antibody to the C-terminus of p53 that 'opens up' p53 into a form competent for sequence specific DNA binding (Hupp *et al.*, 1995). Both the mouse and rat *mdr1b* genes readily bound authentic p53, and binding was specific because it was abrogated by including a specific competitor oligonucleotide containing a consensus p53 site. In contrast, the rat *mdr1b* probe with specific p53 mutations did not bind p53 under any circumstances (Figure 1b).

The functional significance of the rodent *mdr1b* p53-binding sites was determined by assessing whether wildtype or p53 (Figure 1c) activated these promoters in Saos-2 cells that lack endogenous p53. Wild-type p53 activated both rodent promoters and induction was specific as none of the p53 mutants (V143A, R175H, F241S, R273H or D281G) activated transcription.

To test the role of the p53 site in activation of the *mdr1b* promoter, we performed either deletion analysis or the *mdr1b* p53 site was specifically mutated at the positions abrogating binding to purified p53 (Figure 1b). The rat *mdr1b* promoter with an intact p53 site was activated over 15-fold by wt p53 (Figure 2a). As expected, the positive control reporter plasmid (50-2) containing two copies of the muscle-creatine kinase p53 binding site was efficiently activated by co-transfecting the same amount of p53 expression plasmid. Notably, either internal deletion (*Eco/Nde*) or 5′ to 3′ deletion (−162) of the p53 site caused p53-dependent repression of the resulting promoter. Similarly, mutation of the *mdr1b* p53 site caused, not unexpectedly, repression by p53 (Figure 2b). The intact mouse *mdr1b* promoter was readily activated by p53 and its removal, not unexpectedly, caused p53-mediated repression of the promoter (Figure 2c). In summary, the rodent *mdr1b* promoters contain conserved p53 consensus binding sites, bind purified p53 protein *in vitro*, and are transcriptionally upregulated by wt p53 in transient transfection assays *in vivo*.

Hepatic DNA damage induces mdr1b *gene expression and requires p53* in vivo

We determined if regulation of the *mdr1b* promoter by p53 was relevant to regulation of the endogenous *mdr1b* gene in the animal. Acute hepatic exposure to

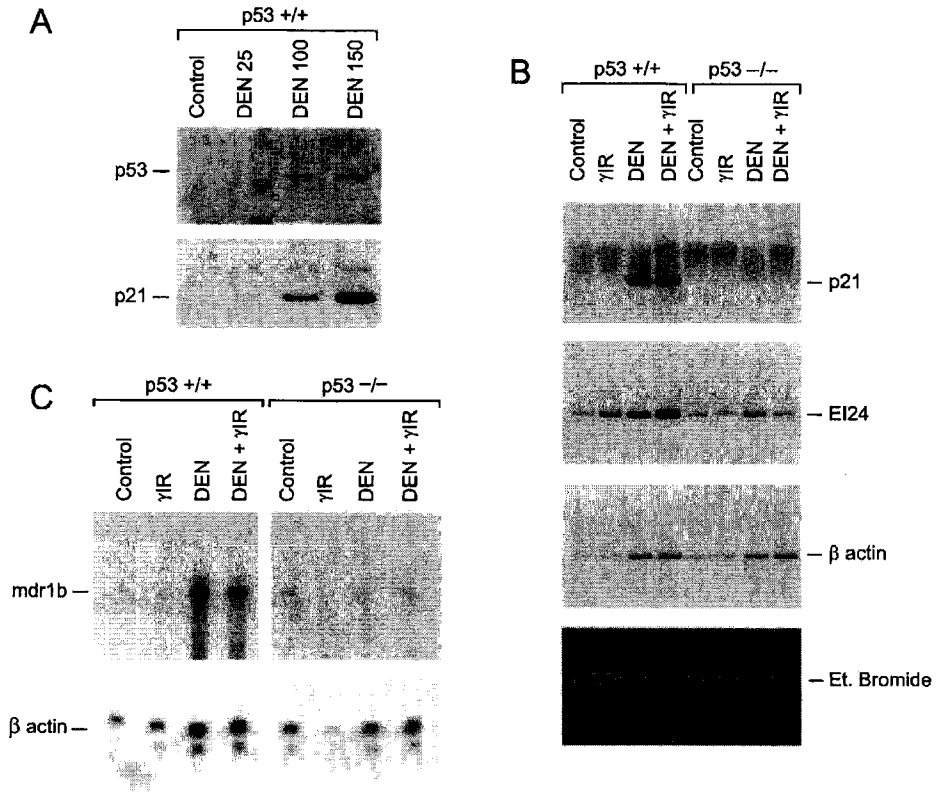

Figure 3 The hepatotoxin diethylnitrosamine (DEN) and ionizing radiation (IR) require p53 to upregulate mdr1b. (**a**) Mice were treated with a single dose of the indicated concentrations of diethylnitrosamine (DEN) (25, 100 or 150 mg/kg) and 24 h later were sacrificed, liver samples isolated and prepared for immunoblot analysis of p53 and p21. (**b**) Northern blot analysis was performed on liver RNA (20 μg) isolated from *p53+/+* or *p53−/−* mice treated with either 150 mg/kg DEN, 10 gray ionizing radiation or a combination of both. The blot was probed, stripped and re-probed successively with cDNAs for *p21, EI24* and *actin*. The ethidium bromide stain is shown to indicate the integrity of the RNA. (**c**) RNAse protection analysis was performed concurrently for *mdr1b* and *actin* on the same RNA samples (15 μg) as described in the Materials and methods

diethylnitrosamine (DEN) ethylates DNA at guanine residues after hepatic P450 metabolism and causes subsequent single-strand breaks (Singh and Roscher, 1991). This form of stress (Levine, 1997) activates p53, a sensor of DNA damage. To determine if DEN induced DNA damage activated p53 *in vivo*, mice were acutely treated with varying doses of DEN, followed by an analysis of hepatic p21 and p53 levels. These studies revealed that hepatic p21 and p53 were induced in a dose-dependent manner (Figure 3a), a finding consistent with the demonstration that DEN damages DNA. Subsequently, p53−/− and p53+/+ mice were treated with 150 mg/kg DEN and/or 10 Gray ionizing radiation (IR), a form of DNA damage that weakly causes p53 activation in the liver of the intact animal (MacCallum *et al.*, 1996). Twenty-four hours after treatment livers were harvested and levels of p53 target genes were determined (Figure 3b). In untreated p53+/+ animals, p21 was undetectable and gamma irradiation caused a small p21 upregulation. In contrast, DEN alone strongly upregulated p21. DEN-mediated upregulation of p21 required p53 as p21 was not induced by DEN in p53−/− mice. Further studies with EI24, a gene originally isolated by differential display from cells undergoing etoposide induced apoptosis (Gu *et al.*, 2000) revealed two interesting findings. First, hepatic induction of EI24 by IR required p53 because EI24 was not induced by IR in p53−/− mice. Second, unlike p21, EI24 was induced by DEN treatment in p53-null liver implying that EI24 responded directly to DEN induced damage.

The role of p53 in the regulation of hepatic mdr1b was evaluated by an analysis of the RNA from the p53+/+ and p53−/− livers of mice treated with either IR, DEN or IR and DEN (Figure 3c). After IR, p21 and *mdr1b* were modestly upregulated in p53+/+ mice (Figure 3b and c). In contrast, DEN strongly increased mdr1b. Interestingly, like p21, mdr1b was not further increased by the combination of DEN and IR. As expected for a gene requiring p53, *mdr1b* was not induced by DEN or DEN plus IR in p53−/− mice. Thus, p53 is essential for the *in vivo* upregulation of murine *mdr1b*.

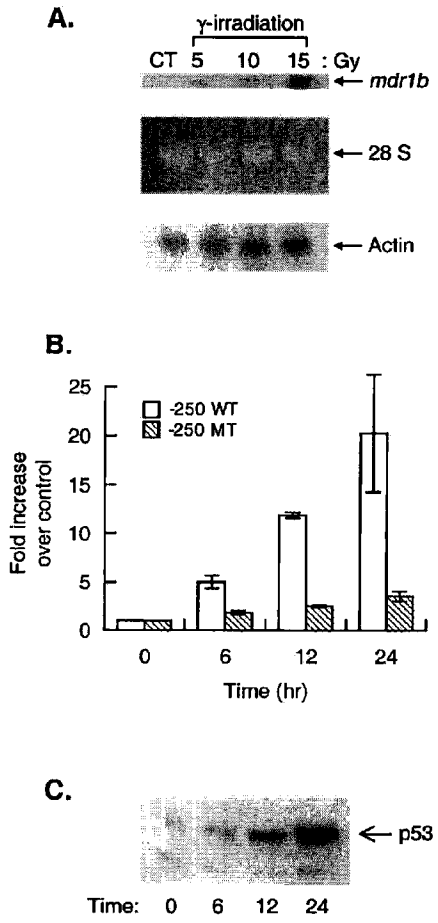

Figure 4 Ionizing radiation induces p53 and activates the *mdr1b* promoter. (**a**) H35 cells were irradiated with the indicated doses, 24 h later RNA was isolated, and analysed by northern blot stained with ethidium bromide for 28S RNA or hybridized with an *actin* cDNA. *Mdr1b* levels were determined by RNAse protection. (**b**) Cells were transfected with either rat *mdr1b* promoter (−250WT) or its p53 mutant (−250MT) reporter. Twenty hours after transfection the cells were irradiated with 10 Gray of γ-IR. (**c**) At the indicated intervals, transfected samples were processed for luciferase activity and p53 expression by immunoblot

Gamma irradiation induces mdr1b expression and activates its promoter

Different forms of DNA damage cause different p53 post-translational modifications with varying potential

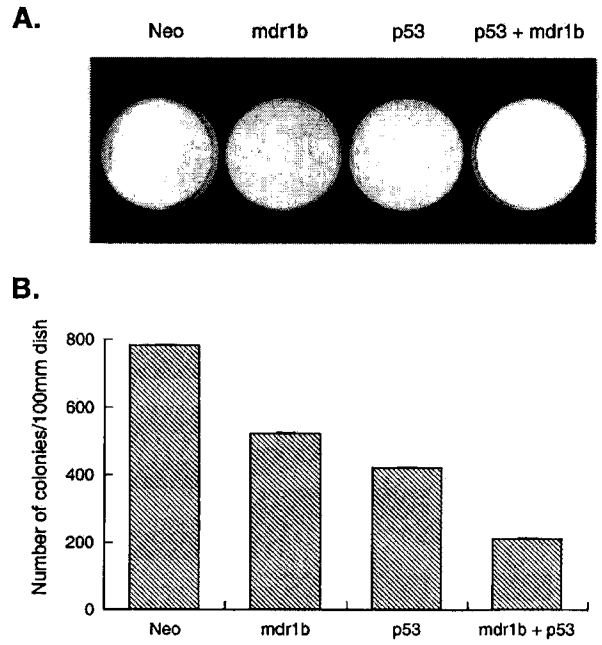

Figure 5 Mdr1b and p53 cooperatively suppress cell growth in Saos-2 cells. Cells were transfected with either pcDNA3-neo (NEO) pcDNA3-mdr1b, pcDNA3 plus the p53 expression plasmid, SN3 or pcDNA3-mdr1b and SN3. The transfected cells were selected in 1.0 mg/ml G418 for 14 days (**a**), stained with crystal violet and (**b**) then enumerated

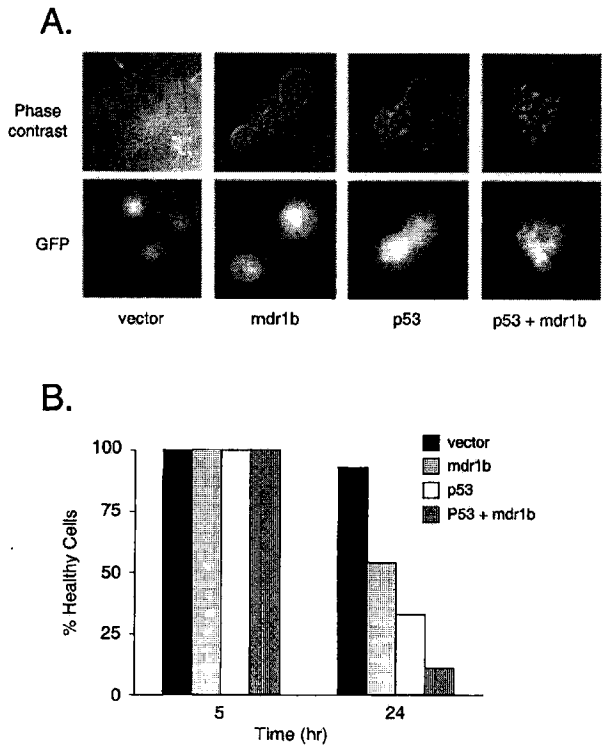

Figure 6 Mdr1b expression enhances p53-mediated decreases in cell survival. COS-7 cells were co-injected with green fluorescent protein (GFP) expression plasmid with either pcDNA3, pcDNA3-mdr1b or the p53 expression plasmid, SN3. The morphology of microinjected cells is shown (**a**). The viability of the cells was monitored and the number of healthy cells was quantified at 5 and 24 h post-injection (**b**). The data shown are one representative experiment out of five comparable independent experiments

to transactivate p53 targets (Giaccia and Kastan, 1998). Ionizing radiation (IR), has not been shown to upregulate mdr1b expression nor activate its promoter. To provide a mechanistic basis for our previous observations (Figure 3), we used the liver cell line, H35 to test this possibility. These cells were irradiated with 5–15 Gray of IR-these doses readily induced p53 and p21 in H35 cells and *mdr1b* levels were determined (Figure 4). Expression of *mdr1b* RNA increased in response to increased IR dose (Figure 4a). To determine if p53 levels correlated with *mdr1b* promoter activation we transfected LS180 cells with the *mdr1b* promoter reporter constructs. After transfection, the cells were irradiated and at intervals simultaneously analysed for both luciferase activity and p53 levels. The transcriptional activity of the *mdr1b* promoter directly correlated with the p53 level (Figure 4c) with a 20-fold increase after 24 h (Figure 4b). In contrast, the *mdr1b* promoter containing specific mutations in the p53 binding site (the same mutations abrogated p53 binding–see Figure 1b) was only minimally increased (Figure 4b) despite the large increase in p53 (Figure 4c). Cumulatively, these studies in both cell culture and the whole animal indicate the *mdr1b* genes contains conserved p53 binding sites that are efficiently and readily activated by p53.

mdr1b *can interact with p53 to decrease cell survival*

Increased expression of mdr1b has been associated with cytotoxic exposure to the topoisomerase inhibitor, mitoxantrone and a non-mdr1 substrate, methylmethane sulfonate, which causes DNA damage after DNA alkylation (Fardel *et al.*, 1998). We therefore reasoned that mdr1b might participate in the cytotoxic response after DNA damage. To assess this possibility we evaluated the effect of mdr1b expression on cell viability using colony assays (Figure 5). The p53-null Saos-2 cells were transfected with either pcDNA3, pcDNA3 plus a p53 expression plasmid, SN3 or pcDNA3 containing the mdr1b cDNA (pcDNA3-mdr1b) or pcDNA3-mdr1b plus SN3. The transfectants were selected in 1.0 mg/ml G418 for 14 days and the colonies were visualized with crystal violet and enumerated (Figure 5a). P53 produced a 46% reduction in the number of colonies and mdr1b also reduced the number of colonies by 30% (Figure 5b). However, mdr1b and p53 reduced colony numbers by 74% suggesting that they cooperate to inhibit cell growth. In support of this concept, transfection of an mdr1b expression vector (pcDNA3-mdr1b) into p53-null Saos-2 or 10(1) cells readily yielded G418-resistant colonies that stably expressed mdr1b (43 and 18%, respectively); in contrast, transfection of the LS180 cell line (which has wt p53 (Thottassery *et al.*, 1997)) revealed only neo-resistant clones that failed to express mdr1b after G418 selection (data not shown). Further, the LS180 cells are not generally impaired for expression of exogenous genes because they readily expressed plasmids containing cDNAs for other genes (not shown).

The reduction in colony numbers by mdr1b overexpression could be a consequence of cell death. To directly test the possibility that mdr1b induced apoptosis, we co-microinjected a green fluorescent protein (GFP) expression plasmid together with either pcDNA3-alone or pcDNA3-mdr1b or the p53 expression plasmid, SN3 alone or SN3 and pcDNA3-mdr1b into the COS-7 cells which are functionally null for p53 (Levine, 1997) (Figure 6). The cells co-injected with cDNAs encoding mdr1b and p53 showed dramatic membrane blebbing and nuclear condensation, and detachment from the culture dish, all characteristics of dying/apoptotic cells (Figure 6a). Besides morphology, we used GFP to quantify the proportion of healthy cells (Figure 6b). These studies demonstrated that those co-injected with mdr1b and p53 had far fewer healthy GFP cells (8%) compared to either mdr1b (52%) or p53 (26%) alone. Cumulatively, these studies indicate that mdr1b cooperates with p53 to decrease cell viability as measured by: (i) decreased colony survival, (ii) changes in cell morphology characteristic of apoptosis (e.g., membrane blebbing, nuclear condensation, detachment) and (iii) decreased number of viable cells expressing GFP.

The colony reduction and microinjection studies demonstrate p53 and mdr1b functionally interact to enhance cell death. To further assess the basis for this interaction we used both acridine orange and ethidium bromide staining of the entire population of cells transfected with various combinations of p53 and/or mdr1b expression vectors (Figure 7a–c). Further, we assessed if p53 transactivation function was required (Figure 7c). Approximately 30 h post-transfection cellular morphology and nuclear condensation were determined (Figure 7a). Those cells with rounded membranes and bright condensed or fragmented nuclei were enumerated as non-viable (Figure 7b,c). The topoisomerase poison and cytotoxin, VP-16, was used as a positive control and it profoundly reduced both the number of cells on the plate as well as the viability of the remaining attached cells. Notably, either mdr1b or p53 alone caused over 50% of the attached cells to appear non-viable. However, unlike mdr1b, p53 appeared to have a greater number of cells still attached (Figure 7b), an outcome that may be attributed to different amounts of p53 affecting either cell cycle arrest or apoptosis (Levine, 1997). When p53 is added to small amounts of mdr1b there is a dramatic enhancement in the loss of attached cells (Figure 7c) and many of the few that remain are apoptotic. To determine if the p53's enhancement of mdr1b mediated cell death required p53's transcriptional function a p53 with impaired transcriptional activation potential was used–p53 with mutations in amino acid 22 and 23 (Roemer and Mueller-Lantzsch, 1996). We found that the apoptosis of mdr1b and 22,23-p53 was not appreciably different than 22,23-p53 alone, but much less that of p53 and mdr1b. This finding demonstrates that increasing amounts of mdr1b enhance p53 apoptosis and this requires, in part, an intact p53 transcriptional activation domain.

Discussion

Previous studies from our laboratory demonstrated that functional inactivation of wildtype p53 upregulates endogenous *MDR1* in human cells and its orthologue, *mdr1a*, in rodent cells, thus demonstrating that wt p53 represses endogenous *mdr1a* expression (Thottassery *et al*., 1997). These results are consistent with others demonstrating elevated MDR1 expression in breast and colon tumors with deletions or mutations in p53 (Ralhan *et al*., 1999). Here we have demonstrated, in both the whole animal and in cells, that *mdr1b* is a downstream target activated by p53. Moreover, rodent *mdr1b* promoters have conserved p53 sites that are required for upregulation of mdr1b after DNA damage. Because some downstream targets of p53 play a role in apoptosis (e.g., BAX, EI24 (Gu *et al*., 2000)) we also determined if mdr1b overexpression affected cell survival. Mdr1b decreased cell survival alone, but when combined with p53, dramatically increased cell death.

The murine and rat *mdr1b* promoters are highly similar (Figure 1) and this sequence conservation extends to their p53 regulation. First, consensus p53 binding sites are positioned at nearly identical distances upstream from transcriptional initiation and are required for *mdr1b* promoter activation by p53. Second, functional p53 is required to transcriptionally upregulate *mdr1b in vivo* and *in vitro*. As *mdr1b* is the more primitive of the *mdr1* genes (as determined by phylogenetic analysis (Growtree) (Furuya *et al*., 1997a)), one interpretation of the current studies is that increased mdr1b decreases cell survival in a p53 and mdr1b dependent fashion. This idea is circumstantially supported by studies showing mdr1b is readily upregulated by cytotoxic agents that are, for the most part, not mdr1 substrates (e.g., 3-methylcholanthracene, aflatoxinB1, methylmethanesulfonate, mitoxantrone or reactive oxygen (Fardel *et al*., 1998; Ziemann *et al*., 1999; Thevenod *et al*., 2000)) and *mdr1b* is transcriptionally upregulated in cells undergoing cell death (Schrenk *et al*., 1996). However, given that different levels of p53 are linked to different cellular outcomes, i.e., low levels of p53 arrest cell growth and high levels induce apoptosis (Levine, 1997); mdr1b's role in apoptosis may be dramatically influenced by the cell context. In addition, other signals (e.g., IL-3) regulate mdr1b expression, and may impact how it functionally affects apoptosis. Moreover, mdr1b has a number of phosphorylation sites which like the cystic fibrosis membrane transregulator may regulate function (Naren *et al*., 1999). Nonetheless, based upon our findings one would predict that in cells that lack mdr1b, p53-dependent apoptosis would be attenuated. Certainly, future studies can only address this issue when *mdr1b* −/− animals become commercially available.

Our data indicate that, besides being a downstream target of p53, mdr1b interacts with p53 in decreasing cell survival, however at this point we cannot quantitatively distinguish synergistic from additive interaction. Nevertheless, in the presence of p53, our studies reveal that mdr1b potently decreased cell survival as supported by the following: (i) dramatic reduction in colony survival in p53-null Saos-2 cells co-transfected with p53 and mdr1b (Figure 4); (ii) enhanced loss of cell viability after co-microinjection of mdr1b and p53 compared to either expression vector alone (Figure 5) and (iii) only cells with no functional p53 (e.g., Saos-2 and 10(1) cells) stably express mdr1b, whereas cells containing functional wt p53 (e.g., LS180) never express mdr1b stably (not shown) and (iv) mdr1b requires a transcriptionally active p53 to enhance the loss of viable cells from the culture. However, because many genes are upregulated by p53 and some in possibly tissue specific patterns, it seems unlikely that upregulation of any one p53 target will be sufficient to trigger apoptosis; thus mdr1b may require additional mediators in effecting cell death, and these may be cell context dependent. Moreover, it is possible that factors that alter mdr1b expression may mitigate some of its apoptotic effects. For instance, preliminary studies, suggest that one of the cytokines that mitigates apoptosis, IL-6 (Yonish-Rouach *et al*., 1991), can

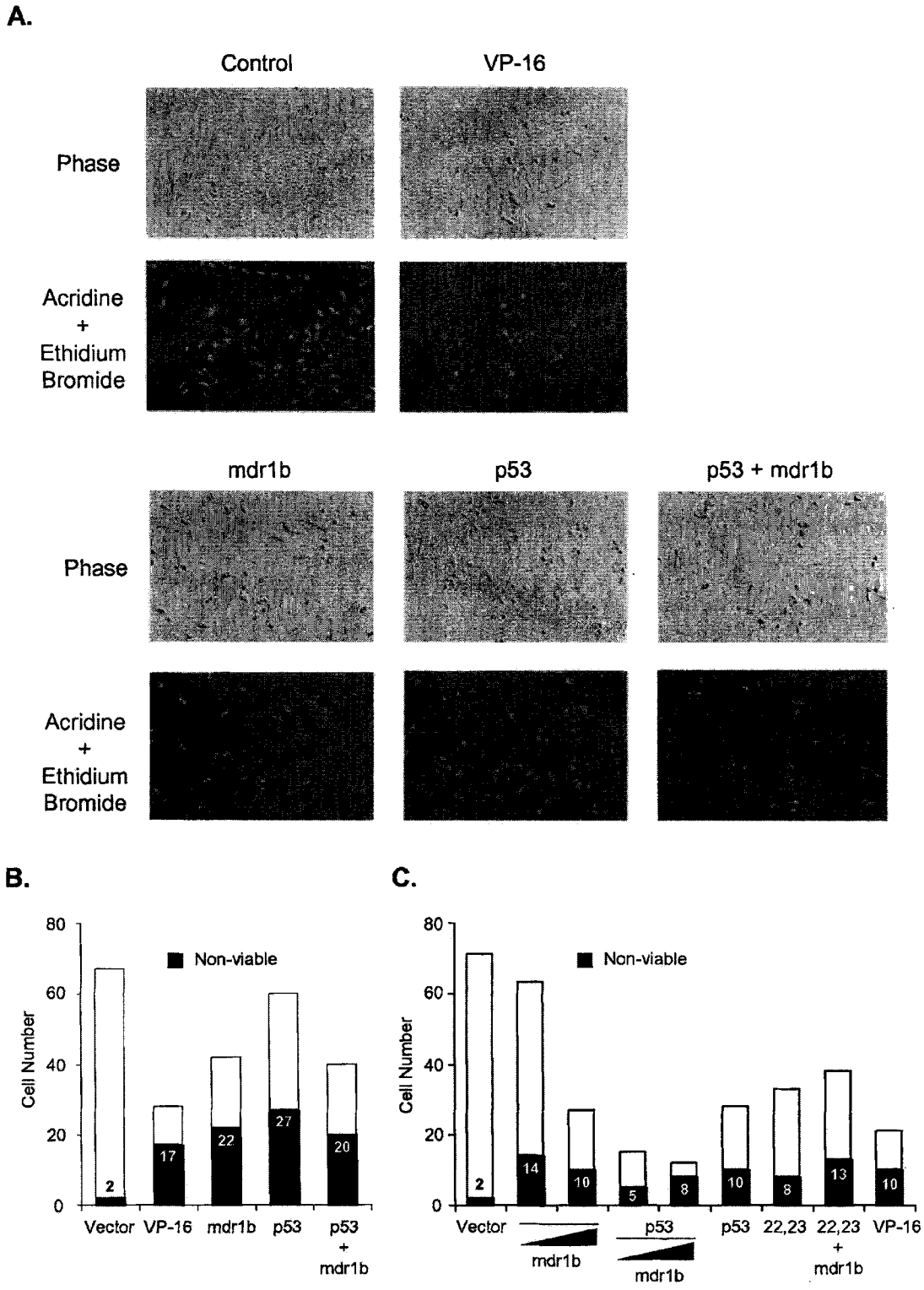

Figure 7 Mdr1b increases p53 mediated cell death and requires an intact p53 transactivation domain. Saos-2 cells were transiently transfected with combinations of pcDNA3 (vector = only this plasmid), pcDNA3-mdr1b or the p53 expression plasmids, SN3, or p53 Gln22, Ser23 or treated with VP-16 (epipidophyllotoxin, 100 μM). Thirty hours later, the cells were stained with acridine orange and ethidium bromide and visually inspected (**a**). The total number of attached cells in a field quantified (white bars) and the cells with both the rounded morphology indicative of apoptosis and green dots characteristic of condensed chromatin and fragmented nuclei (black bars – **b** and **c**). (**b**) Saos-2 cells were transfected with 10 μg of either pcDNA3-mdr1b and 1 μg CMV-NeoBAM (labeled mdr1b), 1 μg SN3 (p53) and 10 μg pcDNA3 (labeled p53) or 1 μg SN3 and 10 μg pcDNA3-mdr1b (labeled p53 + mdr1b).

decrease *mdr1b* promoter activation by p53 (unpublished).

One obvious issue arising from these studies is how does mdr1b affect p53? Both rodent mdr1b and mdr1a transport drugs, however, chloride channel activation in response to osmotic stress is attributed only to mdr1a, but not mdr1b (Valverde *et al.*, 1996; Bond *et al.*, 1998). Thus, we speculate that mdr1b overexpression might provoke a physiological change that enhances p53 function although this has not been proven and is the subject of additional studies. Nevertheless this possibility is not unlikely because alterations in intracellular pH affect the level and consequently function of p53 (Thangaraju *et al.*, 1999). Moreover, a relationship between p53 and ion-channels was demonstrated by recent studies showing potassium channel activation caused apoptosis in a p53-dependent fashion (Chin *et al.*, 1997) and chloride efflux may be critical in the apoptotic cascade (Rasola *et al.*, 1999). Further, a recent study suggests that osmotic alterations enhance p53s function secondary to phosphorylation (Dmitrieva *et al.*, 2000). Certainly future studies will focus on the possibility that mdr1b sends a signal to affect p53's transactivation function.

Materials and methods

Cells and animals

African Green Monkey COS-7, H35 rat hepatoma, and LS180 colon carcinoma cells (Thottassery *et al.*, 1997) (American Type Culture Collection, Rockville, MD, USA) and Saos-2 cells (Dr Jeff Sample, St. Jude) were maintained as indicated by ATCC. Irradiation studies were carried out by treating cells and mice with 10 grays of γ-irradiation (IR) at 325 rads/min using a ^{137}Cs source (JL Shepherd, San Fernando, CA, USA). The mice were treated with a single intraperitoneal injection of diethylnitosamine (DEN) at the indicated concentration. P53 male mice (Taconic Farms, Germantown, NY, USA) were housed in the Animal Facilities at St. Jude.

Transient transfections, reporter assays and plasmids

Cells were transfected by calcium phosphate co-precipitation with 10 μg of reporter plasmid and 100 ng of either p53 expression vector or empty vector. Subsequently the cells were washed twice in phosphate buffered saline and incubated at least 15 min at room temperature with agitation in 400 μL or Reporter Lysis buffer (Promega, Madison, WI, USA) (Thottassery *et al.*, 1999). Luciferase activity was measured in 20–40 μL samples using a Opticomp Luminometer (MGM Instruments, Hamden, CT, USA) and subsequently normalized to protein content. We were unable to normalize to B-galactosidase, chloramphenicol acetyltransferase or renilla activities as p53 represses the traditional viral promoters that drive expression of these control reporters (Subler *et al.*, 1992). The following reagents have been previously described: p50-2 (Zambetti *et al.*, 1992), the wt p53 expression plasmid p53 SN3 (Santhanam *et al.*, 1991), the murine EI24 cDNA (Gu *et al.*, 2000) and p53 Gln 22, Ser23 (Roemer and Mueller-Lantzsch, 1996). The *mdr1b* promoter (−250/+150) was amplified by PCR from a *mdr1b* promoter template (Thottassery *et al.*, 1999) and specific mutations were introduced by overlap extension (Thottassery *et al.*, 1999). The murine *mdr1b* gene was obtained by PCR amplification using the following primers: upstream: 5′-caa aag cag aag cca gta ttc tgcc-3′ and downstream: 5′-gcc aag tgt cca ata cta aaa gtc ccc aac-3′. The murine *mdr1b* promoter sequence matched the reported sequence (Raymond and Gros, 1990) and was subcloned into pGL2 basic (Promega, Madison, WI, USA). The rat *mdr1b* cDNA (kindly provided by Dr Jeff Silverman, AVMAX, San Francisco, CA, USA) was subcloned into pcDNA3 (Invitrogen, CA, USA).

Western immunoblot analysis

Liver was homogenized as previously described in a glycerol-phosphate buffer containing an anti-protease cocktail ('complete' Boeheringer-Mannheim; Thottassery *et al.*, 1997), H35 cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml PMSF and 1 μg/ml aprotinin). Equal amounts of protein (500 μg) were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with a polyclonal anti-p53 antibody (Ab-7) (Oncogene Science, Cambridge, MA, USA). Baculovirus expressed human p53 was used as a positive control (Thottassery *et al.*, 1997). Analysis of p53 in transiently transfected cells was assessed in lysates prepared in 1 × Reporter lysis buffer (Promega, Madison, WI, USA). Analysis of p21 was performed on homogenates of liver with antibody, p21(c-19)-G (Santa Cruz, Santa Cruz, CA, USA).

RNA analysis

Total RNA was isolated from murine liver or H35 cells and analyzed by Northern blot with cDNAs encoding EI24 (Gu *et al.*, 2000) and β-actin. Prehybridization, hybridization with random labeled probes and washing conditions have been previously described (Furuya *et al.*, 1997b).

Ribonuclease protection assay

Rat *mdr1b* was analysed in total RNA by RNAse protection assay using the RPA II kit according to the manufacturer's instructions (Ambion, Austin, TX, USA) and using the Ambion rat *B-actin* antisense probe as a control for RNA integrity. To generate a specific rat *mdr1b* ribonuclease protection probe, the region including codons 643–704 in the rat *mdr1b* open reading frame was PCR amplified from an authentic rat *mdr1b* cDNA, cloned into pCRII.1 (Invitrogen, Carlsbad, CA, USA) and sequenced. The insert was subsequently cloned into pGEM7Z(+) (Promega, Madison, WI, USA) and the T7 promoter was used to direct

(c) Saos-2 cells were transfected with varying amounts of mdr1b (2.5 or 10 μg pcDNA3-mdr1b) in the absence or presence of 1 μg SN3 (p53). Additional cells were transfected with 1 μg p53 Gln22, Ser23 (22,23) in the presence or absence of 2.5 μg pcDNA3-mdr1b (labeled 22+23+mdr1b. All DNA was balanced with either pcDNA3 or CMV-NeoBAM. All experiments were repeated three times with each sample run in duplicate per experiment. At least two different plasmid preparations were used in these studies

synthesis of an antisense *mdr1b* RNA probe. ^{32}P-labeled antisense RNA probes were generated by *in vitro* transcription reactions and antisense RNA hybridized with either 10 or 15 μg of total RNA for 12–16 h. The RNA-RNA hybrids were treated with an RNAse A/T1 mixture, protected fragments separated on a 6% denaturing polyacrylamide gel and visualized by autoradiography. The mouse *mdr1b* RNAse protection probes were kindly provided by Dr Alfred Schinkel (Netherlands Cancer Institute).

Electromobility shift assay (EMSA)

EMSA assays were performed as described previously (Schuetz *et al.*, 1996). One strand of oligoncleotide was end-labeled with γ-^{32}P ATP and T4 polynucleotide kinase. The labeled oligonucleotides were purified by Nuctrap (Stratagene, CA, USA). Labeled oligonucleotides were mixed with poly (dI-dC) (1 μg) in a binding buffer (Gu *et al.*, 2000) and incubated with recombinant human p53 (100 ng) purified from insect cells (greater than 95% pure and labeled, BV) in either the presence or absence of AB421 (2 μg) and/or a p53 consensus site competitor oligonucleotide at 4°C for 30 min. As previously described the monoclonal antibody, pAB421 is required to activate p53 to bind DNA (Hupp *et al.*, 1995). The samples were electrophoresed on a 5% nondenaturing polyacrylamide gel in 0.25 × TBE at 4°C. The synthetic oligonucleotides used in this study were: a p53 consensus site oligonucleotide, 5′-cta gag c gga cat gcc cgg gca tgt ccg cg-3′. The rat *mdr1b* p53 site was: 5′-gaa *catg* tag aga - *catg* tct-3′; For mutant rat *mdr1b* p53 site the italicized bases are critical for p53 interaction (Zambetti and Levine, 1993) were changed from C to T and G to A. The mouse *mdr1b* p53 site was the following: 5′-gaa cac gta aag aca agt cta-3′.

Colony assays

The interaction between p53 and mdr1b was functionally assessed in transfection assays. Cells were transfected with either 10 μg of pcDNA3, pcDNA3-mdr1b, or combinations of 1 μg of pSN3 plus 9 μg of pcDNA3 or plus 9 μg pcDNA3-mdr1b. The cells were selected in 1.0 mg/ml G418 for 14 days, stained with crystal violet and quantified for colony formation with an Arktek colony counter (Thottassery *et al.*, 1997).

Microinjection

Cells were microinjected using a Zeiss inverted microscope with an Eppendorf Transjector (5246) at 40 hPa for 0.4 s and micromanipulator (5171). Cells were plated on glass coverslips 24 h prior to injection in DMEM medium supplemented with 10% FBS. The cells were injected with a green fluorescent protein (GFP) expression plasmid (pGreenLantern, Gibco–BRL) and pcDNA3 (100 ng) or mdr1b-pcDNA3 (100 ng) or a pSN3 (100 ng) expression plasmid, or SN3 and mdr1b (50 ng each), and on average 100–200 cells were injected per sample. Five hours after injection, cells expressing GFP were scored. The number of healthy GFP positive cells were identified by fluoroescence and enumerated. The GFP positive cells were also monitored for morphological changes, which were documented by photography.

Acridine orange/ethidium bromide apoptosis assay

The interaction between p53 and mdr1b in affecting cell viability was evaluated by efficient transient transfection followed by staining with acridine orange and ethidium bromide (Spector *et al.*, 1998). Saos-2 cells (2×10^5) plated on 60 mm dishes were transfected in duplicate with the indicated combination of expression plasmids (mdr1b-pcDNA3 (10.0 or 2.5 μg), and/or SN3 (1.0 μg), and/or p53 Gln22,Ser 23 (1.0 μg)). The total amount of DNA was adjusted to 11 μg of DNA per dish by using either pcDNA3 or CMV-Neo BAM. Approximately −30 h post-transfection the dishes were stained with acridine orange (final concentration 4 μg/ml) and ethidium bromide (final concentration 4 μg/ml). The cells were then monitored for morphological changes indicative of apoptosis i.e., decreased attachment and rounded membranes. The viable cells appeared uniformly green whereas the early apoptotic cells stain green and have bright green intracellular dots indicative of chromatin condensation and nuclear fragmentation. The proportion of cells having both the morphological appearance of apoptosis and the altered nuclear dye-staining were enumerated.

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